OSMOTIC PROPERTIES OF THE ERYTHROCYTE

I. Introduction. A Simple Method for Studying the Rate of Hemolysis

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I.

There is almost no other single type of animal cell which has been so extensively employed in experimental work in the fields of osmotic phenomena and of cell permeability as the mammalian erythrocyte. From the time of the early studies of Hamburger (1886) down to the present day it has been recognized as possessing a number of peculiar advantages as experimental material. Thus, it can be obtained at all times and places in what for practical purposes are unlimited quantities; indeed, the investigator himself carries about with him wherever he goes a never-failing supply of absolutely fresh and normal erythrocytes, ready for use at a moment's notice. Because of the remarkable constancy of its natural environment—the mammalian body—the erythrocyte, unlike certain other cells frequently used for similar studies, may be expected to show only relatively slight variations in its physiological properties from day to day and from season to season. Furthermore, its simple structure and low rate of metabolism prevent complications which are frequently troublesome with other types of material. Removed from the body it can be kept, if not in an unaltered, at least in a usable condition for a longer time than almost any other kind of animal cell. Finally, there are available for its study methods of great simplicity which are not only quantitative but which are also statistical to an extent perhaps nowhere else realized with physiological material.

Because of these striking, and to a considerable extent unique, advantages the erythrocyte would appear to be an almost ideal type of material for studies in which a high degree of quantitative accuracy is desired. A survey of the literature, however, reveals all too frequently a disappointing failure on the part of investigators to obtain results of this character. Not only is there a very common lack of agreement between the conclusions reached by different workers, but even the same investigator is not infrequently forced to acknowledge an inability on repeating his experiments to obtain consistent and reproducible data.

The erythrocyte, in spite of its apparent simplicity, behaves, in fact, as if it were either naturally a highly variable and capricious type of material, or—what is more likely—as if it were peculiarly sensitive to certain environmental factors which with other types of cells are much less troublesome.

In the course of work which has occupied the author for several years and which will be reported in detail in the series of papers of which the present one is the first, the general conclusion has been reached that the erythrocyte is indeed a highly suitable form of material for many types of experimental work and that accurately reproducible results may be obtained with it, but that such results are possible only with a more careful attention to details than is needed with most other forms of physiological material. As a matter of fact, the very simplicity of the mammalian erythrocyte, which in its mature condition is perhaps only questionably to be called a living cell at all, prevents the maintenance by it in a changing environment of the relative internal constancy which is so characteristic of more complicated cells and of entire organisms. The simplicity of the erythocyte is, therefore, rather paradoxically, actually a source of complexity for the experimenter. Furthermore, there are certain special reasons, closely connected with the functions which the erythrocyte has to perform, why its osmotic properties, in particular, are of necessity far more profoundly affected by slight environmental changes than are those of perhaps any other known type of cell. These reasons will be discussed in the second paper of this series.

In general, the relation which the erythrocyte, considered as experimental material, appears to bear to other types of cells is much the same as that which a canoe bears to boats of more stable design. Both the erythrocyte and the canoe when properly handled have very definite and characteristic advantages, but both have the tendency to penalize any carelessness in their management in a prompt and unmistakable manner. Perhaps at some future day this peculiarity of the erythrocyte may be considered rather as an advantage than a disadvantage.

II.

Before considering certain of the peculiarities of the erythrocyte itself it seems advisable to deal with some of the methods which have been employed in the past in studying the osmotic properties of this type of cell, and, in particular, with the one which has been gradually developed by the author and has been used in the experimental work upon which all of the papers of the present series are based. By giving

a single description of the method at this point, unnecessary repetitions may later be avoided.

Osmotic changes in the erythrocyte are, in general, always associated with volume changes. This is true whether the changes are of the simple sort produced by the passage of water alone between the cell and its surroundings or of the more interesting and complicated type, so useful in studies of cell permeability, where the movement of water depends upon osmotic inequalities set up by the passage of dissolved substances across the cell boundary. Any quantitative study of osmotic phenomena will therefore involve the measurement of the amount of volume change which occurs in a given experiment, or the rate of this change, or both.

In the case of the erythrocyte there are available two remarkably simple methods for studying volume changes. The first is the hematokrit method introduced by Hedin (1891). By means of it the total volume of all of the cells in a sample of a given suspension is measured, the cells being tightly packed together in a fine graduated tube by centrifugal force. The advantages of this method are, first, its simplicity and, second, its statistical nature, by which the variability of the millions of individual cells is averaged out. Its greatest disadvantage—and this, unfortunately, is a fatal one in many cases—is that the time required to pack the cells into a mass free from intercellular fluid is so great, even with the most powerful centrifugalization available, that the method can be used only to obtain final end points or, at most, to follow volume changes of extreme slowness. For this reason, in the present series of studies, it has been possible to use it only rarely.

A second method, of even greater simplicity, is that of hemolysis. This method, first systematically employed by Hamburger (1886), depends on the fact that when an erythrocyte in swelling reaches a certain volume, which varies not only with the species of animal but also probably with the individual erythrocyte, it loses a sufficient part of its hemoglobin to become invisible, or almost so, both as viewed singly under the microscope or in the aggregate in a suspension in a test tube. In some cases it is possible by appropriate treatment to restore the invisible corpuscles to visibility; in other cases it is not.

The term hemolysis is sometimes applied to the mere disappearance of erythrocytes; at other times it is used to describe their more complete destruction. This double use of the term, while unfortunate, is perhaps unavoidable at present and every author should therefore designate the sense in which he employs it. It will here be used, for convenience, to apply to what for practical purposes is the easier and more certain

end point to observe, namely, the disappearance of the erythrocyte from visibility rather than its more or less complete destruction, concerning which there is usually much greater uncertainty. This usage is further justified by the fact that in "osmotic hemolysis" complete destruction is apparently very difficult to obtain. Thus, Adair, Barcroft, and Bock (1921) were unable with water alone to separate the hemoglobin from the cells containing it sufficiently to obliterate certain effects believed to be due to the cells themselves, though this could be done after the addition of ether, which presumably completed the destruction of the cells.

The hemolysis method for studying the swelling of erythrocytes and, indirectly, therefore, the penetration of dissolved substances, possesses the advantage of extreme simplicity. With no apparatus other than a test tube, very fair ideas as to many problems of cell-permeability may be obtained. The apparatus here to be described refines the method to an extent which permits the experimenter to secure results of a really high degree of accuracy. An even greater advantage of the hemolysis method, however, is that it is available for the study of *rates* of swelling, even in experiments of very short duration. In the present series of papers no experiments of a total duration of less than one second will be reported, but the author has pointed out elsewhere (1927) that a principle used with conspicuous success for another purpose by Hartridge and Roughton (1923) can be adapted to the study of hemolytic processes whose duration is only a fraction of a second as is the case, for example, with the hemolysis of the erythrocytes of the sheep in distilled water. In its adaptability to problems involving rapid rates of swelling, and consequently some of the most interesting problems of cell physiology, the hemolysis method is, in fact, of unique importance.

On the other hand, the method possesses at least two disadvantages which must be frankly admitted and then dealt with as adequately as circumstances permit. The first is that hemolysis may be caused or influenced by various factors other than osmotic ones. The disappearance of an erythrocyte does not necessarily indicate that it has by swelling reached some definite hemolytic volume, V_h, though this is frequently the case. It is important, therefore, that certain control experiments shall always be performed before inferences concerning the rate of swelling are drawn from observations on the rate of hemolysis.

These control experiments may take various forms. Thus, in cases where osmotic factors alone are involved, it should be possible to show: (1) that the substance or substances present in the solution in which hemolysis occurs have no observable hemolytic effect when added in varying amounts, up to and preferably exceeding those employed in the

experiments, to an isotonic solution of NaCl or some similar non-penetrating substance; (2) that the process of hemolysis by a pure solution of the substance in question may be stopped at will at any desired point by the addition in osmotically suitable amounts of NaCl, saccharose, etc.; or (3) that if a solution of NaCl be chosen which is sufficiently hypotonic to cause the hemolysis of some but not all of the erythrocytes in a given sample of blood, the addition of the substance to the partially hemolyzed suspension causes no increase in the degree of hemolysis. The last mentioned test is a very delicate one, though it is somewhat difficult to employ for reasons to be discussed in the following paper of this series.

A second disadvantage of the method is that even in cases where it is reasonably certain that the occurrence of hemolysis is due to the attainment of a definite volume, V_h, this volume represents merely one point on the swelling curve. As compared with the egg of Arbacia (Lillie, R. S., 1916), (McCutcheon, M., and Lucké, B., 1926) whose volume changes can be measured continuously, the erythrocyte appears capable at best of supplying to the investigator only very meagre information about the course of the swelling process.

This disadvantage, however, is not so serious as it might at first sight appear to be. There is reason to believe that the course of the swelling of the erythrocyte can be represented by a fairly simple equation (Jacobs, M. H., 1928) which permits the entire curve to be calculated approximately when one point on it is known. This question will be dealt with more fully in a later paper. Furthermore, in perhaps most experiments, what is desired is not so much the entire curve of swelling as some general measure of the velocity of the swelling process under various experimental conditions, and this may frequently be obtained by a comparison of the times required under the conditions in question to reach the *same* state of swelling in each case. For work of this type the critical hemolytic volume, V_h, when such a volume exists, is a very satisfactory and convenient criterion for comparison.

One important additional point connected with the use of the hemolysis method remains to be mentioned. Both this and the hematokrit methods are statistical in the sense that millions of cells are employed with each. But whereas the latter measures the total volume of all of the cells together without separating them into groups, the former is complicated by the fact that different individual cells hemolyze with different degrees of readiness, and in determining the time of hemolysis, the cells must, in effect, be divided into groups for separate time-measurements. The size of these groups will depend upon the delicacy of the method employed. When a distinction can be made between, for

example, 75 per cent and 76 per cent apparent hemolysis, as is the case with the method about to be described, then the time of hemolysis for the group of cells lying between these limits and consisting of one per cent of the total number may be taken as approximately the arithmetical mean of the times at which the above-mentioned degrees of hemolysis are attained. With a cruder method, or in the region of five or ten per cent hemolysis, where measurements are much more difficult to make, the groups dealt with are of necessity larger and a mere averaging of two times gives correspondingly less accurate results.

Because of the heterogeneous nature of any collection of erythrocytes, it is impossible to speak simply of the "time of hemolysis" for a given sample of blood. Different times must be measured for different groups of cells, or, if desired, a single group may be arbitrarily selected for a given experiment by determining in advance for what particular degree of hemolysis the time shall be measured. In any case, the problem is a much more complicated one than if the blood contained only erythrocytes of uniform physiological properties.

On the other hand, a certain degree of heterogeneity may in some respects be an advantage. Assuming that the different degrees of osmotic resistance of the various cells are dependent chiefly on different individual values of the critical hemolytic volume, V_h, which is a plausible, though as yet an entirely unproved assumption, a possible means is suggested for obtaining more information about the course of the entire swelling curve than could be furnished by a perfectly homogeneous group of cells. The details of such a method still remain to be worked out.

A much more definite advantage of the heterogeneity of a given population of erythrocytes is the following. It is frequently necessary to find a solution of "critical concentration" for a group of cells, *i.e.*, which is just at the point of being able to hemolyze these cells without actually doing so. Cells in such a solution are extremely sensitive test objects for studying the effects of such factors as pH, temperature, etc., as will be pointed out in greater detail in a later paper. If the cells in such a group possessed identical properties, it would require many trials to find the appropriate concentration to the desired degree of accuracy (i.e., to less than 0.001M). With as heterogeneous a group, however, as the erythrocytes in ordinary blood, any concentration within fairly wide limits may be selected with the certainty that there will be present in the blood a group of cells which will exactly "fit" the concentration so chosen. In later papers frequent applications of this principle will be mentioned.

III.

A method suitable for the study by the hemolysis method of the osmotic properties of the erythrocyte should possess the following characteristics. It should allow the degree of hemolysis to be estimated more accurately and more rapidly than the usual laborious and not very exact methods of making cell counts or of making hemoglobin determinations after a preliminary centrifugalization. It should permit the time required for the attainment of a given percentage of hemolysis to be measured accurately, even when the total duration of the experiment is only a few seconds. The usual methods are entirely useless in such cases, and this is perhaps the reason why little work has as yet been done on the rates of any except very slow types of hemolysis. The method should, in the third place, provide not merely for the measurement of the time required to reach some single percentage of hemolysis but for that required for the attainment of many different percentages; otherwise, the heterogeneous nature of a population of erythrocytes may give rise to a type of difficulty that will be discussed in a later paper. Finally, though less essential than the characteristics already mentioned, simplicity of the apparatus itself and convenience in its use would be highly desirable features.

The method here described possesses all of these characteristics. It permits successive determinations of the relative concentrations of cells in different suspensions, as well as of apparent percentages of hemolysis, to be made in a few seconds each, which under favorable conditions are reproducible to one or two per cent. It may be used for the study of all rates of hemolysis where the time measured is more than one second. Furthermore, it permits the measurement not merely of the time required to reach some arbitrarily selected degree of hemolysis but also of the times corresponding to all percentages from zero to upwards of 90 per cent. These measurements, which are extremely easy to make, take the form of permanent kymograph tracings where mistakes in instrumental readings or in the recording of them by the observer are impossible, and where all of the details of the experiment are presented in a way that facilitates ready interpretation. Finally, the apparatus is very simple and inexpensive. A crude but satisfactory form of it can be constructed in an hour out of materials available in any laboratory, and its operation can be mastered in a few minutes. The variety of uses to which it can be put and the degree of accuracy which can be secured with it will be made more evident in the later papers of this series.

In principle, the method is not new. It involves merely the measurement of the turbidity of a suspension of erythrocytes by determining

the maximum depth of the suspension through which the image of the glowing filament of a carbon lamp is visible. It is to be noted that what is observed is a distinct image rather than the total amount of transmitted light, as is the case, for example, with the methods of Ponder (1923, 1927) or with the nephelometer. Methods similar to the present one for the study of suspensions have been used or suggested by Vlès (1921), Holker (1921) and others, but they lack certain of its most useful features.

The source of the image is the filament of an old-fashioned carbon lamp. The brightness of the filament is kept constant by the use of a milliammeter to measure and a sliding rheostat to regulate the current flowing through it. For the particular lamp employed, a current of 200 milliamperes has proved to be a suitable one and has been everywhere used except where otherwise specified. If desired, the depth of the suspension may be kept constant and the current measured which under the given conditions makes the filament visible. This method, however, is inferior to the one adopted in being less sensitive and in involving more difficult calibrations.

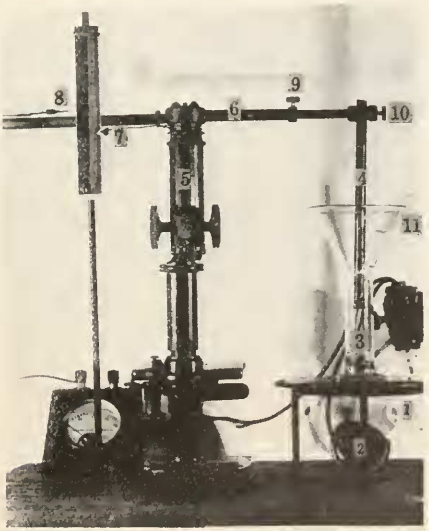

FIG. 1. A simple form of the apparatus, described in detail in the text.

A form of the apparatus somewhat simpler than the one actually employed, but which shows more clearly in a photograph its most essential parts is illustrated in Fig. 1. The image of the filament, 1, is reflected upward by the mirror, 2, through the vessel, 3, in which the

suspension to be examined is placed. This vessel is in the form of a tube 2.5 cm. in diameter with a funnel-like expansion above and closed below by a glass plate cemented to the tube with deKhotinsky cement. In cases where it is not necessary to keep the whole apparatus in a water bath for temperature control a separate glass funnel may be substituted.

Into the vessel, 3, plunges a tube, 4, coated internally with a dead-black varnish and closed at its lower end by a small coverglass cemented to it with deKhotinsky cement. It is best always to cover with paraffin any such cement which can come in contact with the solutions used in the experiments. The position of the plunger, 4, is adjusted by means of the rack and pinion of an ordinary microscope, 5, to the tube of which it is attached by the arm, 6. Attached to the microscope are also the pointer, 7, which gives readings on a millimeter scale and the writing point, 8, which touches the smoked paper of a kymograph (not shown). Any movement of 4 is therefore recorded by the kymograph, while at the same time its exact setting can be read from the scale. At the beginning of an experiment the apparatus is adjusted so that a scale reading of zero corresponds to close contact between the bottom of 3 and that of 4. The necessary adjustments of 4 are facilitated by the screws, 9 and 10. Where greater simplicity is desired, a satisfactory substitute for the arm, 6, can be improvised from several ordinary metal clamps.

Since in osmotic experiments on the erythrocyte (as will be pointed out elsewhere) accurate temperature control is essential, the vessel, 3, is usually immersed almost to the top of the funnel in a covered water-bath (not shown in Fig. 1) with blackened interior to cut off all light except that passing through a glass window in its bottom. For the design of this water-bath and for several other features of the apparatus the author is indebted to his assistants, Mr. Arthur K. Parpart and Mr. Wilbur A. Smith.

When the apparatus has been set for a series of experiments it is desirable not to disturb it in changing solutions. This is easily avoided by emptying the vessel, 3, through a removable glass tube (not shown) attached to a filter pump. Another fine-pointed glass tube, also not shown, is usually allowed to dip into the solution in 3. This tube is connected with the compressed air supply and provides in short experiments for rapid and uniform mixing of the blood and the solutions introduced into 3, while in longer ones the current of air may be used as desired to prevent any settling of erythrocytes on the bottom of the vessel. Other tubes connected with the compressed air supply and also not shown provide for the stirring of the water in the water-bath and the prevention of condensation of moisture on the window in its bottom when it is employed at low temperatures.

In using the instrument in an ordinary hemolysis experiment, the procedure is as follows. The tube, 4, is elevated until it is considerably above the position of the expected initial reading. The desired quantity of blood (usually one carefully formed drop from a special pipette) is placed on a small removable paraffin-coated shelf, 11, which is suspended from the side of the funnel. The kymograph is started and the compressed air turned on. Then, as the solution is suddenly poured upon the blood with one hand, the tube is lowered by the other until the image of the filament just appears. The beginning of the experiment is therefore shown by a sudden drop in the line made by the writing point. When the image is seen to increase slightly in brightness, the tube is quickly raised a few millimeters, causing it to disappear. When it again appears the tube is again raised, and this process is repeated until the tube emerges from the liquid. With the apparatus employed by the author and with 25 c.c. of liquid, which is a convenient quantity, this occurs at a scale reading of approximately 60 mm. representing, for samples of blood, in the proportions used, between 80 and 90 per cent apparent hemolysis.

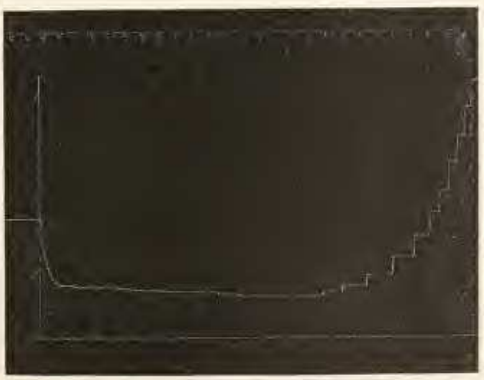

FIG. 2. Typical record of the course of hemolysis of ox blood in 2M ethylene glycol. The time intervals are 5 seconds with every twelfth signal omitted.

The type of record obtained in an experiment of this sort is illustrated in Fig. 2. This particular record gives 11 points on a curve representing hemolysis of ox blood in 2M ethylene glycol. The time intervals marked on the record are of five seconds each with every twelfth one omitted. The slow fall of the curve prior to the sudden rise which indicates hemolysis is due to the gradual recovery by the erythrocytes, with the penetration of the solute, of their initial volumes, and their subsequent further swelling, after a pronounced shrinking

has been produced by the concentrated solution employed. Even with solutions of penetrating substances isosmotic with blood, the swelling that precedes hemolysis is usually indicated by a slight fall in the curve. By using greater dilutions of blood so that the readings appear higher on the scale these effects can be considerably magnified and used to

FIG. 3. Typical record of the partial hemolysis of ox blood in 0.082M NaCl. The marks on the curve indicate 30 second intervals.

good advantage in studying volume changes rather than hemolysis. As would be expected, swollen corpuscles produce lower and shrunken ones higher readings than normal ones, a fact already noted by Holker (1921).

In experiments of longer duration, where the method described is wasteful of kymograph paper and fatiguing to the eye of the observer, it is preferable to make readings at regular intervals marked by the writing point itself, allowing the drum to move only enough each time to record the level of the reading. A record of this sort covering 18.5 minutes with readings every 30 seconds is reproduced in Fig. 3. It represents the partial hemolysis of ox corpuscles in 0.082M NaCl slightly buffered for pH 7.4 with phosphates.

When the duration of the experiments is very short, *i.e.,* less than perhaps 10 seconds, kymograph records become difficult to make. Fairly complete and accurate hemolysis curves may be obtained, however, in such cases by setting the instrument in advance at any selected point and determining with a stop-watch the time required to reach this point. The vessel is then emptied and the experiment repeated with a different setting of the instrument, and so on, as many times as desired. The complete curve may then be plotted from the separate points obtained.

With experiments of such extremely short duration (*i.e.* less than

perhaps 1.5 seconds) that the time required for the uniform mixing of the blood and the solution becomes significant, it is scarcely profitable to attempt to obtain times corresponding to the lower scale readings. Fair accuracy, however, may be secured with sufficiently high settings so that most of the suspension is under the bottom of the inner tube, in which case imperfect mixing is much less serious than otherwise. It is for this reason, as well as because of the fact that the accuracy of the instrument is greater for the higher scale readings, that the author has chosen 75 per cent apparent hemolysis of an approximately 1 : 500 suspension as a very convenient criterion for comparison when for any reason it is necessary to select some single degree of hemolysis for this purpose. With the apparatus used and with most samples of blood this point usually corresponds to a scale reading in the vicinity of 40 mm.

In the use of the instrument several precautions may be mentioned. The only subjective feature of the method is the decision by the observer as to when the filament may be said to be visible. This decision is made with different degrees of readiness and constancy by different persons. The author finds it most convenient so to place the lamp and the mirror that what is seen in the tube is a single small loop of the filament. A reading is taken when the exact form of the entire loop is visible. To secure the greatest sensitiveness of the eye, readings should always be approached from the side of the invisibility rather than from that of the visibility of the filament. In any case, it is important to work fairly rapidly. The image should be approached without hesitation and the reading made without an attempt by moving the tube up and down unnecessarily to secure exactly the right degree of distinctness. What might otherwise be gained in this way is more than lost by the changes that are caused in the sensitiveness of the eye of the observer.

In general, the experience of each individual will soon teach him under what conditions he can secure the most reproducible results. Fortunately, the method permits no possible bias to enter into the measurements, since the observer is unable while making a reading to see the record on the drum, which reproduces with strict fidelity the results of his judgment. It is therefore a very simple matter for anyone using the method to obtain in this way, on a drum moved for the purpose by hand, a series of readings at different levels, which, when subsequently measured, will give exact information as to the reliability of his readings. The readings of the author, in a test of this sort, rarely show a variation of more than 0.3 mm. for a scale reading of 10 mm. or of more than 1.5 mm. for a scale reading of 50 mm. For a sus-

pension whose initial reading is 10 mm. these variations correspond to differences in the estimated percentages of hemolysis of approximately three and less than one per cent, respectively. By averaging a number of readings for a single point, such errors can be still further reduced. The method is therefore seen to be capable of yielding results of a high degree of accuracy.

IV.

The question of the relation between the observed scale readings and the corresponding degrees of hemolysis may now be discussed. Changes in the opacity of the suspension are due primarily to changes in the number of cells which it contains and secondarily to changes in

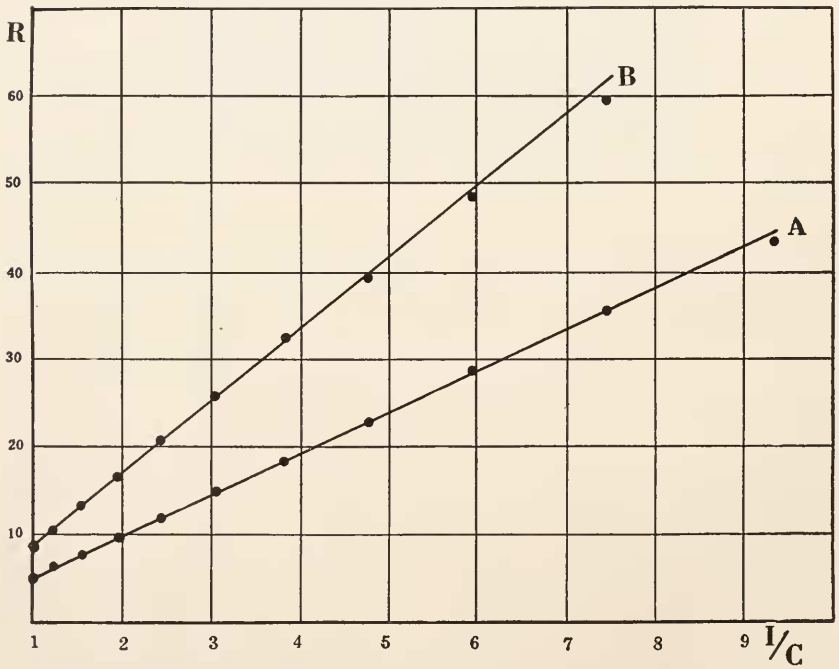

FIG. 4. Effect of dilution of blood on scale reading. Curve *A* represents dilution with 0.9 per cent NaCl of a suspension of ox erythrocytes. Curve *B* represents dilution of a similar but originally less concentrated suspension (approximately 1:500) with a solution containing hemoglobin in the proper amount to give standards representing different degrees of apparent hemolysis; $R =$ scale reading in millimeters and $1/C =$ reciprocal of concentration in arbitrary units.

the properties of the individual cells and of the surrounding medium. Since the first mentioned factor is by far the most important, it may be considered first by itself as uncomplicated by, for example, the state

of swelling of the cells or the presence of hemoglobin in the surrounding solution.

The relation between the concentration of cells in a given suspension and the scale reading of the instrument may readily be obtained by a simple calibration experiment in which a geometrical series of dilutions of an original suspension is used for purposes of standardization. For example, beginning with 125 c.c. of a fairly concentrated suspension of cells in 0.9 per cent NaCl, 25 c.c. are removed for the first measurement and are replaced by 25 c.c. of the salt solution. After thorough mixing this process is then repeated for any desired number of times, a series of suspensions each four-fifths as concentrated as the one preceding it being obtained. Frequently, a factor of dilution of three-fourths or even one-half will give results which are entirely satisfactory with correspondingly less labor.

If now the scale readings so obtained are plotted against the reciprocals of the concentrations, as has been done in the graph labelled *A* in Fig. 4, it will be seen that the points lie almost on a straight line, indicating that the relation between the scale reading R, and the concentration C may be represented approximately by the rectangular hyperbola,

$$CR = \text{a constant.}$$

Actual calculations show that the errors introduced by estimating the relative number of cells in a given suspension, as compared with a standard, by means of this simple relation are usually insignificant. Thus, Table I, from which the data used in constructing graph *A* of Fig. 4 were obtained, shows in columns 1 and 4, respectively, the relative concentrations of cells as determined by actual dilution and as calculated from the relation,

$$CR = R_0,$$

R_0 being the scale reading for the original suspension whose concentration is taken as unity.

It will be observed that the differences amount in no case to more than one per cent, though in other similar experiments differences of two per cent, or rarely more, have been obtained. In general, the differences are greater for low scale readings where the errors of observation are relatively large. Because of the important effect of slight errors in determining the initial scale reading, R_0, an average value for the constant in the equation may, if desired, be obtained from all of the CR products. For comparison with the figures already mentioned there are given in column 5 concentration values calculated in this way. On the whole, they are seen to agree very closely with the values in columns 1 and 4.

As to the simple mathematical relationship found to exist between the number of cells and the observed scale-reading, it may be stated that much the same relation has been reported by Vlès (1921) and Holker (1921) who used methods somewhat similar in principle to the present one, though differing from it in a number of respects, for measuring the opacity of various cell suspensions.

TABLE I

Relation between Scale Readings and Concentration of Erythrocytes in Suspensions

Concentration in arbitrary units = C	Scale readings in millimeters = R (each figure is the average of 10 readings)	Product CR*	Concentration calculated from initial reading R_0 = 5.0	Concentration calculated from average of CR products = 4.85
1.00	5.0	5.0	—	—
.80	6.3	5.0	.79	.77
.64	7.7	4.9	.65	.63
.51	9.6	4.9	.52	.51
.41	12.0	4.9	.42	.40
.33	15.0	5.0	.33	.32
.26	18.3	4.8	.27	.27
.21	22.6	4.7	.22	.21
.17	28.6	4.8	.17	.17
.13	35.3	4.7	.14	.14
.11	43.6	4.7	.11	.11

* This product was calculated from more accurate values of C than those in column 1, which are rounded off to two places of decimals only.

The exactness with which relative numbers of cells can be estimated from scale readings, either by calculation or by the use of appropriate standards, particularly for readings above 20 mm., suggests the possibility of using the apparatus, though it was designed primarily for studies of hemolysis, for making the ordinary red-cell counts so frequently needed in physiological and in medical work and for which the laborious and not very accurate hemocytometer method is commonly employed. Preliminary experiments in this direction have shown that by first diluting the blood so that the resulting suspension gives a reading on the more sensitive part of the scale, successive independent determinations differing from one another by no more than one or two per cent may be obtained at will. The time required for each determination, exclusive of that required for cleaning and drying the blood pipette is approximately 15 seconds. With the enormously more laborious hemocytometer method, successive counts, as is well known, usually vary by at least five per cent. Of course, the method gives only relative and not absolute numbers of cells (though it can be made

absolute within the limits of the hemocytometer method itself by means of one preliminary cell count) and the readings obtained with it are affected by any variation in the size and shape of the erythrocytes in different samples of blood, as well as by their numbers. The errors to be expected from these sources, however, under the usual physiological conditions are not likely to be as great as those constantly and unavoidably associated with the far more difficult method now almost universally employed.

In using the instrument to estimate percentage of hemolysis, several factors in addition to the concentration of cells must be considered. In the first place, during hemolysis not only do the cells decrease in number, but the hemoglobin liberated from them and contained in the surrounding solution absorbs light and therefore tends to produce lower scale readings than correspond to the mere number of cells. This complication may be dealt with readily, however, by making the dilutions in the calibration series with a solution containing the concentration of hemoglobin that would result from complete hemolysis of the cells. Such a solution is readily prepared by adding to distilled water twice the quantity of blood contained in the same volume of the standard suspension and then, after complete hemolysis has occurred and the solution is entirely transparent, mixing with it an equal volume of sodium chloride solution of twice the concentration of that desired.

As a matter of fact, it turns out that with the dilution of blood that is otherwise most convenient to work with (approximately one part of blood to five hundred of solution) the effect of the hemoglobin on the reading of the instrument, while detectable, is, practically, almost negligible. Under these circumstances the product:

$$(100 - \text{per cent hemolysis}) \times \text{scale reading}$$

proves to be almost constant, as is indicated in graph B of Fig. 4 where the scale readings plotted against the reciprocals of the percentage of unhemolyzed cells lie almost on a straight line.

The theoretical apparent percentages of hemolysis represented in the prepared standards in this particular experiment and the corresponding figures as calculated by the equation

$$\text{per cent hemolysis} = 100 \left(1 - \frac{R_0}{R}\right)$$

are given in Table II in columns 1, 3 and 4, respectively, and are seen to be in better agreement than might, from the nature of the case, reasonably have been expected.

For many purposes, therefore, with a very fair degree of accuracy,

apparent percentages of hemolysis may simply be calculated from initial scale readings as if the presence of hemoglobin in the external solution could be disregarded. For such calculations, a graphic method, which perhaps requires no explanation here, has been found to save much time. In cases where higher concentrations of erythrocytes are employed or where special accuracy is required, however, appropriate standards for calibration should be prepared.

TABLE II

Relation between Scale Readings and Apparent Percentages of Hemolysis

Apparent percentage of hemolysis represented by standard	Scale reading (each figure is the average of 5 readings)	Percentage hemolysis calculated from initial scale reading	Percentage hemolysis calculated from average of *CR* products
0	8.6	—	—
20	10.4	17	20
36	13.0	34	36
49	16.6	48	50
59	20.6	58	60
67	25.7	67	68
74	32.2	73	74
79	39.2	78	79
83	48.5	82	83
87	59.6	86	86

The assumption has so far tacitly been made that a given per cent of hemolysis may be represented by a mixture of unaltered cells and of completely hemolyzed cells. This is, unfortunately, not strictly true. In the first place, any solution which is sufficiently dilute to cause osmotic hemolysis of any of the cells must of necessity cause swelling of all of the unhemolyzed cells. In the second place, the possibility must be considered that cells which have not as yet undergone hemolysis may have, nevertheless, given up some of their hemoglobin to the surrounding solution. Both of these factors might be expected to have optical effects which would considerably complicate the situation as so far outlined.

With regard to the first factor, an approximate allowance may readily be made for it by taking as the initial reading for purposes of calculation, not that for a given suspension in 0.9 per cent NaCl, but that for a similar suspension in a solution which is decidedly hypotonic though not quite sufficiently so to cause any hemolysis. This concentration may readily be determined by experiment; for ox blood it is usually in the vicinity of *M*/8 NaCl. Figures obtained in this way by calculation or by calibration with standards made up as before but with the use of hypotonic instead of isotonic solutions, are undoubtedly

more accurate than those secured with the neglect of this precaution. It is impossible, however, because of the heterogeneous nature of the material dealt with, to prepare by a simple method of mixtures standards which reproduce with complete fidelity the conditions in a partly hemolyzed sample of the blood.

Even more troublesome is the second difficulty mentioned above. If osmotic hemolysis is, as is maintained by Saslow (1929) an "all or none" phenomenon, then the preparation of standards representing fairly well a given degree of hemolysis is perhaps possible. If, on the other hand, as is believed by Báron (1928), this is not the case, but in a given mixture of hemolyzing cells some have undergone complete hemolysis (in the sense of becoming completely invisible), while others have lost lesser amounts of hemoglobin which can be expected to vary greatly with the conditions of the experiment, then not only is it impossible to prepare standards representing accurately different percentages of hemolysis, but the term percentage of hemolysis itself ceases to have any very exact meaning.

Under these circumstances, and until there is more general agreement than there is at present as to whether osmotic hemolysis is or is not an "all or none" phenomenon, it is perhaps unprofitable to try to introduce into our methods refinements which may have little real significance. It seems preferable merely to speak, as has already been done, of an apparent or an approximate percentage of hemolysis, using for our estimations some convenient though arbitrary type of standard. Figures of this sort will have a considerable value, if used with a recognition of their limitations. In any case, regardless of the type of standard employed, such figures will usually involve an uncertainty of only a few per cent in the assumed degree of hemolysis.

In the absence of any general agreement at present as to a precise definition of percentage of hemolysis, the especial value of a method such as the one here described becomes apparent. The kymograph tracings obtained with it are exact and unequivocal. There may be doubt as to whether a certain point on the record indicates 75 per cent or 78 per cent hemolysis, but the point itself is not in doubt. In most experiments what is desired is not so much to know how long it requires to reach, for example, exactly 75 per cent hemolysis, assuming that this expression has any precise meaning, but rather how long it requires under the chosen conditions to reach a point on the hemolysis curve which can be represented by some reproducible standard. This is possible with the present method with a high degree of accuracy.

Summary

1. A simple method is described by which it is possible to measure with a very satisfactory degree of accuracy the rate of hemolysis where the time involved exceeds approximately one second. If the duration of the experiment is ten seconds or more, a complete graphic record of the entire process up to an apparent degree of hemolysis of between 80 and 90 per cent may be obtained.

2. The method may also be used for the accurate determination of the relative numbers of erythrocytes in different suspensions and, assuming a satisfactory definition for the expression "percentage of hemolysis," for the rapid estimation of the latter, within the range most useful for experimental purposes, with an error of no more than one or two per cent.

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